Immunology of onchocerciasis. I. Association of antigenaemia with depression of delayed cutaneous hypersensitivity

M. Arango*, E. Lugo*, I. des Moutis, A. Caprón** y L. Yarzábal***

*Centro Amazónico para Investigación y Control de Enfermedades Tropicales "Simón Bolívar" (CAICET). Puerto Ayacucho. Venezuela. **Centre d'Immunologie et de Biologie Parasitaire. Institut Pasteur. Francia.

Inmunología de la oncocercosis (I). Asociación entre antiginemia y ausencia de reacciones cutáneas de hipersensibilidad retardada

En veintinueve individuos (27 de ellos, Yanomani Amerindians) seleccionados al azar de entre una población de 428 pacientes afectos de oncocercosis, residentes en zonas endémicas de Venezuela, se investigó la presencia de antígenos circulantes de *Onchocerca volvulus* mediante una prueba de radioinmunodifusión (RIPEGA), utilizando inmunoglobulinas de conejo específicas contra un extracto antigénico de *O. volvulus* marcadas con ^{125}I. Dicho extracto que se obtuvo por digestión con colagenasa de los parásitos procedentes de nódulos subcutáneos fue absorbido con un antisuero de caballo antiproteínas humanas para eliminar las posibles proteínas del huésped. Los antisueros obtenidos (a partir de 6 conejos) eran capaces de reconocer 14-16 componentes antigénicos del parásito y 1-2 componentes séricos humanos: estas reactividades contra componentes del huésped fueron eliminadas por absorción de la fracción aislada de inmunoglobulinas (Ig anti-*O. volvulus*) con suero humano normal. Con este preparado de Ig anti-*O. volvulus* se identificaban 1-2 bandas de precipitación (por doble inmunodifusión) en los sueros de pacientes con oncocercosis, siendo elegida para su marcaje con ^{125}I y posterior uso en el RIPEGA, aquella fracción de Ig anti-*O. volvulus* que ofrecía bandas de precipitación con un mayor número de sueros.

El 63 % de los pacientes del grupo de Yanomani Amerindians residentes en las zonas endémicas, presentaban antígenos circulantes. Se halló además que la antiginemia estaba significativamente correlacionada con la negatividad de las pruebas cutáneas de hipersensibilidad retardada frente a un extracto soluble de *O. volvulus*: sólo un 10 % de los pacientes con antígeno circulante, mostraban reacciones cutáneas positivas de hipersensibilidad retardada (HR), frente a un 63 % de pacientes sin antiginemia con reacciones cutáneas HR positivas. Es posible que exista una correlación entre microfilariodermia y antiginemia ya que los únicos 3 individuos, cuyas biopsias cutáneas no revelaban la presencia de microfilarias mostraron resultados negativos en la prueba de RIPEGA, mientras que el 70,8 % de los que presentaban microfilarias en la piel daban resultados positivos. No existió correlación entre el nivel de antiginemia y la presencia de anticuerpos anti-*O. volvulus*.

This study considers the presence of detectable circulating antigens in patients with onchocerciasis and its relationship to cutaneous hypersensitivity. Twenty-nine individuals were randomly selected from a population of 428 living in an endemic zone of onchocerciasis in Venezuela; 27 of these were Yanomami Amerindians.

Polyvalent rabbit antisera were raised using a preparation of *Onchocerca volvulus* made by homogenizing worms extracted by collagenase digestion from nodules; this preparation was absorbed with an antihuman antiserum to remove host protein. This antisera were found to recognize 14-16 parasite-derived antigenic components and 1-2 human components; these antihuman reactivities were removed by absorption with normal human sera. Antisera, raised against the insoluble fraction of the parasite extract produced 1-2 precipitation bands in 6 to 15 onchocerciasis sera tested. Using these sera with a radioimmunodiffusion assay (RIPEGA), it was found that 63 % of the Yanomami group living in the endemic zone carried circulating antigen (antigenaemia). The presence of antigen in the circulation was found to be associated with negative delayed hypersensitivity reactions as measured by an intradermal test with a crude soluble extract of *O. volvulus*. Only 10 % of the patients with circulating antigen, gave positive delayed skin responses compared with 63 % of those without circulating antigen. There was no correlation between the antigenaemia level and the level of specific anti *O. volvulus* antibodies. A possible correlation between microfilarial skin load and antigenaemia may exist since the three individuals without microfilariae in the skin gave negative results in RIPEGA test while 70,8 % of those with microfialarodermia gave positive results.

INTRODUCTION

Two distinct clinical types have been identified in onchocerciasis: localized infection in which lesions are limited to one limb accompanied with a low number of microfilariae in the skin[1], and generalized infections which present multiple cutaneous, subcutaneous and ocular lesions associated with elevated parasite loads[2].

Correspondencia: L. Yarzabal. Inmunología Clínica, Apartado 50647 Correos de Sabana Grande. Caracas. Venezuela.

From the immunological point of view it has been observed that in the localized form, immediate and delayed hypersensitivity reactions are manifested *in vivo* against antigenic extracts of *Onchocerca volvulus*, while in the generalized form the T cell-mediated immunological response is depressed in a high percentage of cases[3,4].

Burtlett et al[3], postulated that this immune depression may be due to the action of antiparasite antibodies, suppressor cells or substances of parasite origin. Ouaissi et al, and Des Moutis et al[5,6], have demonstrated circulating antigens in onchocerciasis In the present study, we report preliminary results which show an association between the presence of circulating antigens and suppression of delayed cutaneous hypersensitivity in onchocerciasis patients.

MATERIAL AND METHODS

Human sera

Included in this study were 29 sera selected randomly from 428 specimens collected between May and November 1981, from the Yanomami Amerindian and North American communities in Parima B, Venezuela (02°55'N, 64°14'W; altitude 950 m above sea-level), the composition of the group being 27 Yanomami and 2 resident north americans; sera from 17 europeans living in areas free from onchocerciasis were used as controls. Samples of blood were collected by venipuncture. Coagulation and retraction was at ambient temperature for 6-18 hours. The sera were filtered through Millipore membranes of 5 µm pore-size, aliquoted, maintained at 4° C for 1-3 days and then stored at –20° C until use..

Preparation of antigen from *O. volvulus*

Antigenic extracts were prepared from *O. volvulus* for use in intradermal tests and in the preparation of polyvalent immunosera for radioimmunoassays. Adult filariae were isolated from subcutaneous nodules removed surgically from Yanomami in the Sierra Parima. The nodules were submerged immediately after removal in liquid nitrogen and stored at –196° C until use. Nodules were thawed for the preparation of antigen and then washed three times at ambient temperature in physiological saline containing 200 units/ml of penicillin and 200 µg/ml of streptomycin. They were then placed in flasks of 125 ml capacity containing 20 ml of sterile medium 199, pH 7.2, with 200 units/ml penicillin and 200 µg/ml streptomycin and 4 mg/ml collagenase (Sigma type IV, 125 units/mg) and incubated at 37° C with gentle agitation (30 rpm). The medium was changed every 12 hours until complete digestion of host tissue was achieved. The free worms were washed 6 times in physiological saline and extracted overnight at 4° C in 1:1000 saline solution and then homogenized at 4° C. The homogenate was passed 4 times through a hydraulic press (Wabash, 6.000-8.000 psi) at 4° C and centrifuged at 3.500 g for 1 hour at 4° C. The supernatant and the pellet were dialyzed separately against 3 changes of 500 volumes of distilled water at 4° C and then lyophylized. Before use the fractions were resuspended in physiological saline and sonicated for 4 periods of 20 seconds at maximum power (Niveau ultrasonicator) and then absorbed with horse antihuman antiserum to remove any remaining host proteins[7].

Preparation of polyvalent anti-*O. volvulus* antisera

These were prepared according to Vaitukaitis et al[8]. Six adult rabbits were immunized, 2 with the absorbed supernatant preparation, 2 with the absorbed sediment preparation and 2 with a mixture of equal parts of the two preparations. Each animal, received an injection of 2 mg of material in 1 ml of physiological saline emulsified with 1 ml of Freund's complete adjuvant. Samples of blood were collected at 10,20 and 30 days following injection and the immunoglobulin fraction separated from the serum by ammonium sulphate precipitation[9]. This was then purified further by immunoabsorption with normal human serum.

Testing and labelling of polyvalent anti-*O. volvulus* antisera

Aliquots of each of the purified immunoglobulin preparations were lyophilized for testing with double immunodiffusion in agar[10] against normal human sera and 15 sera from onchocerciasis patients. Those immunoglobulin preparations producing bands against the greatest number of onchocerciasis sera were labelled with ^{125}I by the chloromine T method[11].

Radioimmunoprecipitation with polyethylenglycol (RIPEGA)

The method of Santoro et al[12] was used. The concentration of polyethylenglycol (PEG) required to precipitate only immune complexes (5 %) was determined previously. For the test 0.2 ml of polyclonal antibodies labelled with ^{125}I (30.000 cpm) was added to 0.2 ml of serum from each patient diluted previously 1:5 in borate buffer (0.1 M, pH 8.4). After 2 hours incubation at 37° C and 6 hours at ambient temperature, the circulating antigen and labelled antibody complexes were precipitated with 5 % PEG. Each test was made in triplicate and the results are expressed as the percentage of ^{125}I labelled anti-*O. volvulus* polyclonal antibodies precipitated in relation to the radioactivity bound to proteins precipitated with 20 % trichloroacetic acid.

Investigation of cutaneous reactivity

After the collection of a sample of blood, each onchocerciasis patient was given 4 intradermal injections of 0.1 ml of each of the following: *a)* a crude soluble extract of *O. volvulus* from the Sierra Parima at 40 µg/ml (preparation A); *b)* the same extract at a concentration of 20 µg/ml (preparation B): *c)* a crude, soluble extract of *Ascaris suum* at 20 µg/ml (preparation C), and *d)* phenol saline solution used as the solvent for the antigen preparations. Measurements of cutaneous reactions were made at 15-30 minutes (immediate hypersensitivity) and at 48 hours (delayed hypersensitivity). The areas of immediate reactions were recorded on graph paper and were considered as being positive, when the papular area was equal to or greater than twice the area of the reaction against phenol saline solution. The size of delayed reactions was measured by taking the perpendicular diameters of the area of induration and

TABLE I Correlation between antigenaemia and delayed cutaneous hypersensitivity

Antigenaemia	Number of patients	Delayed cutaneous hypersensitivity against			
		O. volvulus (40 µg/ml)		*A. suum*	
		+	−	+	−
≤ 11.75 %	8	5 (63 %)	3 (37 %)	2 (25 %)	6 (75 %)
> 11.75 %	10	1 (10 %)	9 (90 %)	0 (0 %)	10 (100 %)

those with average diameter of greater than 5 mm were considered as being positive reactions.

ELISA tests

The presence and concentration of circulating anti-*O. volvulus* antibodies, was determined by the enzyme-linked immunosorbent assay (ELISA) as described by Yarzábal et al[13]. Soluble *O. volvulus* antigen at 15 µg/ml was used in the tests and 25 of the 27 sera from the Yanomami subpopulation were assayed.

RESULTS

Characteristics of the anti-*O. volvulus* polyvalent antisera

Immunological analysis of the antisera obtained by immunization of rabbits against extracts of *O. volvulus* revealed antibodies against 14-16 parasite immunogens and 1-2 components of normal human serum; the latter were removed by absorption with human serum. The antisera raised against the absorbed sediment preparations produced 1-2 precipitation bands against 6 out of 15 onchocerciasis sera, and were subsequently labelled and used in this study. The antisera against the mixture of sediment and supernatant reacted with only 3 of the sera and those against the supernatant produced no precipitation bands.

Prevalence of antigenaemia

To establish the limits of significance of the results obtained from radioimmuno-precipitation with PEG the results from the control group were analyzed. This group consisted of 17 europeans and gave a mean value of 7.9 ± 1.9 %. Reactions greater than 11.7 % (the mean plus two standard deviations) were thus considered as being positive.

The group of patients produced results varying between 8.0 % and 23.2 % (mean value – 14.2 ± 4.7 %) with all of the positive results being in the Yanomami subgroup. In the Yanomami subgroup 17 of the 27 sera gave RIPEGA reading greater than 11.7 % which is equivalent to a prevalence of antigenaemia of 63.0 % of the population. No difference was evident between the sexes with 4 of 6 women (66.7 %) and 13 of 21 men (61.9 %) producing positive results. All of the 17 control subjects and the 2 north americans included in the study produced negative results.

TABLE II Correlation between antigenaemia and microfilarodermia

With microfilarodermia * (n) Antigenaemia (% ± SD)	Without microfilarodermia * (n) Antigenaemia (% ± SD)
(24) 14.93 ± 4.56	(3) 8.53 ± 0.72

* (n): Number of patients.

Correlations between antigenaemia and cutaneous reactivity

Of the 29 individuals resident in the hyperendemic area from whom serum was collected, 28 were tested for cutaneous reactivity to antigen preparations from *O. volvulus* and *A. suum*. In 18 of these individuals readings were taken at both 15-30 minutes and at 48 hours while only immediate reactions could be measured in the other 10.

All of the 18 patients gave positive immediate reactions against both antigens, 33 % showed delayed-type hypersensitivity against *O. volvulus* antigen and only 25 % exhibited delayed cutaneous reaction against *A. suum* antigen. When a comparison was made between the delayed cutaneous reaction and the level of antigenaemia it was observed that only 10 % of the patients with circulating antigen produced a positive response to *O. volvulus* antigen in contrast to 63 % of those without circulating antigen (table I). This difference was significant ($\chi^2 - 28.61$; $p < 0.001$).

Correlation between antigenaemia and the microfilarial load in the skin

An examination of the clinical history of the 29 residents in the hyperendemic area revealed that 25 (86.2 %) had microfilariae in biopsies of skin with a mean density ranging from 3.6 to 1055.3 mf/mg of skin and an average level of 36.0. In the Yanomami subgroup there were 24 positive (88.9 %) with a mean density of 36.0 mf/mg o skin. One north american produced a positive results with 3.6 mf/mg of skin. In a comparison between the level of antigenaemia and the presence of microfilarodermia in the Yanomami subgroup it was seen that the three individuals without mf in the skin gave negative results in RIPEGA (table II) where as 70.8 % of those carrying mf gave positive results with an average level of antigenaemia of 14.9 %.

TABLE III Correlation between antigenaemia and anti-*O. volvulus* antibodies

Antigenaemia	Number of patients	Min-max	Results from ELISA	
			Mean	Standard desviation
≦ 11.75	10	1:3200-1:12800	1:5440	3334
> 11.75	15	1:800-1:25600	1:6453	5611

Correlation between antigenaemia and the presence of anti-*O. volvulus* antibodies

Of the 27 sera collected from the Yanomami 25 were examined by ELISA for the presence of anti-*O. volvulus* antibodies. All gave positive results with titres between 1:800 and 1:25.600. No significant correlation could be detected by an analysis of variance between the titre of antibody and the level of antigenaemia (table III).

DISCUSSION

The preliminary results presented in this communication demonstrate that the depression of delayed cutaneous hypersensitivity observed in Yanomami Amerindians infected with *O. volvulus* is associated with the presence of circulating parasite antigen. The interaction of those substances with populations of host immunocompetent cells could explain the alteration of the T-lymphocyte mediated response detected in generalized forms of onchocerciasis. Pérez-Rojas et al[14], studying the same indigenous group of the Sierra Parima as in the present study observed an inhibitory effect of onchocerciasis serum on the capacity of normal lymphocytes to proliferate in culture in response to mitogens or alloantigens. Haque et al[15], have demonstrated that the serum of patients suffering from generalized from of onchocerciasis contains factors capable of inhibiting the reactivity of mouse spleen cells or normal human lymphocytes against T and B mitogens.

Parasite products with similar activity have been described by Capron et al[16], and Dessaint et al[17], in culture fluids of *Schistosoma mansoni* and by other authors in soluble extracts of *Trypanosoma gambiense* and *Trichinella spiralis*[10]. Circulating onchocercal antigens have recently been demonstrated by Ouiaissi, et al[15] and des Moutis et al[16], in human onchocerciasis. Such substances could act by suppressing the function of T and B subpopulations as seems to be the case with antigens of *S. mansoni*[16] or perhaps through lymphocytotoxic mechanisms as occur with extracts of *T. spiralis*[18].

On the other hand, *O. volvulus* antigens could exercise their depressive effect on the T-response in association with the corresponding circulating antibodies in the form of circulating immune-complexes (CIC). Such complexes have been observed in onchocerciasis patients[19-23]. Steward et al, and are capable of blocking at central and peripheral levels the cellular arm of the immune-response[24, 25]. This blocking effect of CIC has been demonstrated by our group in paracoccidioidomycosis, a fungal infection which also produce a depression of delayed cutaneous hypersensitivity[26, 27].

In onchocerciasi, as in paracoccidioidomycosis, parasite antigens alone or in association with antibodies could reduce cellular immunity through a blocking of the surfase receptors of T-lymphocytes or by the stimulation of a T-suppressor population.

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